

ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING

5

EXPRESSION OF TNF- α

CROSSREFERENCE TO RELATED APPLICATIONS

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This application claims priority from United States
Provisional Application 60/062,718.

BACKGROUND OF THE INVENTION

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1. FIELD OF THE INVENTION

The present invention provides antisense
oligodeoxynucleotides targeted to exon sequences flanking
donor splice sites which regulate expression of TNF- α .

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2. DESCRIPTION OF RELATED ART

There has been increasing interest in the
development of antisense oligodeoxyribonucleotides (AS-
ODNs hereinafter) as therapeutic agents and experimental
tools (Stein and Cheng, 1993; Wagner, 1994). However,
25 despite the improvement in affinity for target RNA,
increased resistance to nucleolytic cleavage, and

enhanced delivery of AS-ODNs to cells and their nuclei
(Hodges and Crooke, 1995), high concentrations of AS-ODNs
continue to be required to inhibit gene expression. To
some extent, high AS-ODN concentrations have hampered the
5 development of this technology as an effective
pharmacological agent because of cost and non-specific
AS-ODN actions.

Many genes encode pre-mRNAs containing introns that
are removed by a splicing process that is directed by a
10 complex of small nuclear ribonucleic proteins (snRNPs)
called the spliceosome (Staley and Guthrie, 1998).
Several reports indicate that gene expression is
effectively inhibited by AS-ODNs targeting the
intron/exon boundaries of splice sites (Boeve and De Ley,
15 1994; Dominski and Kole, 1996; Dominski and Kole, 1994;
Hodges and Crooke, 1995; Moulds et al., 1995), likely
because these domains direct splicing events (Staley and
Guthrie, 1998). It has previously been shown in cell
free systems that the degree of sequence variability at
20 splice sites influences splicing events (Dominski and
Kole, 1994), suggesting that pre-mRNAs with variant
splice site sequences would be ideal targets for AS-ODN
treatment (Hodges and Crooke, 1995). Since exon
sequences upstream of donor (5'), and downstream of
25 acceptor (3') splice sites within pre-mRNA play a
critical role in processing RNA (Staley and Guthrie,

1998), it is plausible that these sites encode RNA domains highly susceptible to AS-ODN-mediated inhibition of gene expression. To date, this hypothesis has not been tested systematically in a biologically relevant system such as tumor necrosis factor alpha (TNF- α) production in cell culture or *in vivo*.

Under normal conditions, TNF- α 's expression is tightly regulated by rapid mRNA turnover (Gearing et al., 1995). However, in disease states, its expression is perturbed, resulting in overexpression (Sharief and Hentges, 1991; Tracey and Cerami, 1994). TNF- α is implicated in the pathogenesis of several inflammatory diseases including multiple sclerosis (MS) (French-Constant, 1994), rheumatoid arthritis (RA) (Lupia et al., 1996), viral infections such as human immunodeficiency virus (HIV) (Fauci, 1996) and, bacterial infections causing sepsis (Tomioka et al., 1996). TNF- α neutralizing antibodies (Givner et al., 1995), soluble TNF- α receptors (Moreland et al., 1997), or gene knockouts of the TNF receptor (p55) (Pfeffer et al., 1993) mitigate the harmful effects of TNF- α observed in several animal models of inflammation (Probert et al., 1995; Selmaj et al., 1991). However, these approaches do not limit TNF- α synthesis.

Several studies show that AS-ODNs targeting TNF- α mRNA limit TNF- α synthesis (Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996). However, in these reports, concentrations of AS-ODNs in excess of 2 μ M, were required to achieve significant inhibition. High concentrations of AS-ODNs may induce non-specific inflammatory cell responses (Hartmann et al., 1996) as well as other non-specific effects (Gao et al., 1992; Khaled et al., 1996; Perez et al., 1994). Nevertheless, earlier reports suggest that expression of other genes can be regulated by low concentrations (≤ 1 μ M) of AS-ODNs (Hanecak et al., 1996; Miraglia et al., 1996). Therefore it would be useful to develop AS-ODNs that can be used in low concentrations to regulate TNF- α production in inflammatory responses.

SUMMARY OF THE INVENTION

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According to the present invention, a synthetic nuclease resistant antisense oligodeoxynucleotide capable of selectively modulating expression of human tumor necrosis factor-alpha by targeting exon sequences flanking donor splice sites, thereby regulating

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expression of TNF- α in a patient in need of such therapy is provided. In an embodiment either AS-ODN having the sequence set forth in SEQ ID No:4 or SEQ ID No:6 or a combination thereof can be used. The AS-ODN is
5 administered either as the active ingredient in a pharmaceutical composition or by utilizing gene therapy techniques as an expression vector.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when
15 considered in connection with the accompanying drawings wherein:

FIGURE 1 is a bar graph which shows AS-ODNs targeting exon sequences flanking the donor splice site of exon 2 and 3 of TNF- α effectively inhibit TNF- α
20 protein production. Percent reduction of TNF- α levels in PMA/PHA stimulated U937 cells treated with AS-ODNs targeting various domains within the TNF- α open reading frame. PMA/PHA stimulated U937 cells produced 750 ± 75 pg TNF- α /ml/million cells. * ($p \leq 0.01$) ** ($p \leq 0.001$).

25 **Unstim-** Unstimulated U937 cells; **O-1 through O-21-** ODNs

complementary to different sequences of TNF- α . Data are presented as a mean \pm SD (n=3).

FIGURE. 2A-B are bar graphs which show ORF4 (SEQ ID No:4) and ORF6 (SEQ ID No:6) reduce TNF- α production in a dose-dependent manner. (FIGURE 2A) U937 cells were treated with ORF4 or ORF6 (1 μ M, 100 and 10 nM) and supernatant TNF- α levels were measured by ELISA. (FIGURE 2B) AS-ODNs are not cytotoxic to U937 cells. Data are presented as a mean \pm SD (n=3). * ($p \leq 0.01$) ** ($p \leq 0.001$).

FIGURE 3 is a bar graph which shows ORF4-PE dose-dependently reduces TNF- α mRNA in stimulated U937 cells.

RT-PCR was used to detect TNF- α and GAPDH mRNA levels in U937 cells treated with ORF4-PE. Densiometric analysis of TNF- α RT-PCR products from U937 cells treated with ORF4-PE. Relative TNF- α mRNA levels were calculated based on the pixel density ratio of TNF- α :GAPDH PCR product in each separate reaction. Data are presented as a mean \pm SD (n=3). * ($p \leq 0.05$) ** ($p \leq 0.01$).

FIGURE 4A-B are bar graphs which show ORF4-PE specificity for TNF- α . (FIGURE 4A) U937 cells were treated with ORF4-PE and supernatant TNF- α and IL-6 levels were measured by ELISA. (FIGURE 4B) ORF4-PE-mediated inhibition of mitogen-induced TNF- α gene

expression in primary human PBMC, macrophages and murine monocytes. PMA/PHA stimulated PBMC produced 1250 ± 110 pg TNF- α /ml/million cells. LPS-stimulated primary macrophages produced $13,500 \pm 1,700$ pg TNF- α /ml/million cells. LPS-stimulated murine monocytes produced $7,100 \pm 875$ pg TNF- α /ml/million cells. Data are presented as mean \pm SD (n=3). * (p \leq 0.01) ** (p \leq 0.001).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a synthetic nuclease resistant antisense oligodeoxynucleotide (AS-ODN) capable of selectively modulating human tumor necrosis factor alpha (TNF- α) by targeting exon sequences flanking donor splice sites thereby regulating expression of TNF- α in a patient in need of such treatment. Donor splice sites represent the 3' end of an exon and are located at the junction between the exon and intron.

By modulating/regulating it is meant that the expression of TNF- α is inhibited or reduced by the action of the AS-ODNs.

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In an embodiment either:

SEQ ID No:4 CTG ACT GCC TGG GCC AGA GGG CTG ATT AG

SEQ ID No:6 CCA CAT GGG CTA CAG GCT TGT CAC TCG

can be used or any combination thereof.

5 As shown in the Example hereinbelow, SEQ ID No:4, when made nuclease resistant by phosphorothioate bonds linking between the four 3'-terminus nucleotide bases, is effective and non-toxic.

Since exon sequences are critical in mRNA processing
10 (Dominski and Kole, 1996; Dominski and Kole, 1994) and genes with short internal exons, such as TNF- α , are highly susceptible to exon skipping (Dominski and Kole, 1991), as shown herein it appears that TNF- α 's internal exons encode domain is highly susceptible to AS-ODN
15 actions. As shown herein: (a) the inhibitory action of AS-ODNs targeting TNF- α exon sequences upstream of donor sites and downstream of acceptor sites as well as AS-ODNs that target other regions of the TNF- α gene were determined; (b) stringent criteria in the design and
20 selection of each AS-ODN was used for maximizing its potential efficiency and; (c) AS-ODNs developed by the above criteria are shown effective in different cell types in which TNF- α was stimulated by different signaling pathways. AS-ODNs targeting exon sequences
25 flanking the 2nd or 3rd exon donor splice sites

significantly inhibited TNF- α protein production.

Therefor exon sequences flanking donor splice sites of the small internal exons of TNF- α are domains that are highly susceptible to the AS-ODN treatment of the present invention.

The present invention provides pharmaceutical compositions as described hereinbelow and gene therapy means of administering the AS-ODN of the present invention to regulate TNF- α expression. The active ingredient of the pharmaceutical composition is at least one synthetic nuclease resistant antisense oligodeoxynucleotides, or ribozymes, targeting exon sequences flanking donor splice sites, such as SEQ ID No:4 OR SEQ ID No:6 in a physiologically acceptable carrier or diluent. The concentration range of the AS-ODN in the pharmaceutical composition is generally 1.0 μ M to 100nM.

Phosphorothioate antisense oligonucleotides do not normally show significant toxicity and exhibit sufficient pharmacodynamic half-lives in animals [Agarwal et al., 1996]. Antisense induced loss-of-function phenotypes related with cellular development were shown for the glial fibrillary acidic protein (GFAP), for the establishment of tectal plate formation in chick and for the N-myc protein, responsible for the maintenance of

cellular heterogeneity in neuroectodermal cultures
(epithelial vs. neuroblastic cells, which differ in
their colony forming abilities, tumorigenicity and
adherence). Antisense oligonucleotide inhibition of a
5 basic fibroblast growth factor (bFgF), having mitogenic
and angiogenic properties, suppressed 80% of growth in
glioma cells [Morrison, 1991] in a saturable and specific
manner. Being hydrophobic, AS-ODN interact well with
phospholipid membranes [Akhter et al., 1991]. Following
10 their interaction with the cellular plasma membrane, they
are actively (or passively) transported into living cells
[Loke et al., 1989]

The term "oligodeoxynucleotide" refers to an
oligomer or polymer of nucleotide or nucleoside monomers
15 consisting of naturally occurring bases, sugars and
intersugar (backbone) linkages. The term also includes
modified or substituted oligomers comprising non-
naturally occurring monomers or portions thereof, which
function similarly. Incorporation of substituted
20 oligomers is based on factors including enhanced cellular
uptake, or increased nuclease resistance and are chosen
as is known in the art. The entire oligodeoxynucleotide
or portions thereof may contain the substituted
oligomers.

Instead of an antisense sequence, as discussed herein above, ribozymes may be utilized for suppression of gene function. This is particularly necessary in cases where antisense therapy is limited by stoichiometric considerations [Sarver et al., 1990, Gene Regulation and Aids, pp. 305-325]. Ribozymes can then be used that will target the same sequence. Ribozymes are RNA molecules that possess RNA catalytic ability [see Cech for review] and cleave a specific site in a target RNA. The number of RNA molecules that are cleaved by a ribozyme is greater than the number predicted by stoichiometry. [Hampel and Tritz, 1989; Uhlenbeck, 1987]. Therefore, the present invention also allows for the use of the ribozyme sequences, targeted to exon sequences flanking donor splice sites, which regulate expression of TNF- α expression and contain the appropriate catalytic center. The ribozymes are made and delivered as discussed herein below. The ribozymes may be used in combination with the antisense sequences.

Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA

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(sTRSV) (Sullivan, 1994; U.S. Patent No. 5,225,347, columns 4-5). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication. Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). The ribozyme type utilized in the present invention is selected as is known in the art. Hairpin ribozymes are now in clinical trial and are the preferred type. In general, the ribozyme is from 20-100 nucleotides in length.

Nuclease resistance, where needed, is provided by any method known in the art that does not substantially interfere with biological activity of the antisense oligodeoxynucleotides or ribozymes as needed for the method of use and delivery [Iyer et al., 1990; Radhakrishnan, et al., 1990; Eckstein, 1985; Spitzer and Eckstein, 1988; Woolf et al., 1990; Shaw et al., 1991]. As shown herein in the Example, ORF4-PE (a phosphorothioate derivative of ORF4; SEQ ID No:4) is a preferred embodiment.

Modifications that can be made to antisense oligonucleotides and ribozymes in order to enhance nuclease resistance include modifying the phosphorous or oxygen heteroatom in the phosphate backbone, short chain

alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. These include preparing methyl phosphonates, phosphorothioates, phosphorodithioates and morpholino oligomers. In one
5 embodiment, it is provided by having phosphorothioate bonds linking some or all the nucleotide bases.

Phosphorothioate antisense oligonucleotides do not normally show significant toxicity at concentrations that are effective and exhibit sufficient pharmacodynamic
10 half-lives in animals [Agarwal et al., 1996] and are nuclease resistant. Other modifications known in the art may be used where the biological activity is retained, but the stability to nucleases is substantially increased. The efficiency of inhibition and toxicity can
15 be tested as shown herein in the Example to determine the most effective nuclease resistant protocol.

The nuclease resistant AS-ODNs of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical
20 condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such
25 considerations as are known in the art. The amount must

be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, lower levels of expressed mRNA for TNF- α or improvement or elimination of symptoms and other indicators as are
5 selected as appropriate measures by those skilled in the art. General methods of administration are provided herein which can be modified as known in the art to accommodate the requirements of maintaining and delivery of AS-ODNs.

10 Once the nuclease resistant oligonucleotide sequences are ready for delivery they can be introduced into cells, as is known in the art. Transfection, electroporation, fusion, liposomes, colloidal polymeric particles and viral vectors, as well as other means known
15 in the art, may be used to deliver the oligonucleotide sequences to the cell. The selected method depends on the cells to be treated and the location of the cells and will be known to those skilled in the art. Localization can be achieved by liposomes, having specific markers on
20 the surface for directing the liposome, by having injection directly into the tissue containing the target cells, by having depot associated in spatial proximity with the target cells, specific receptor mediated uptake, viral vectors, or the like. Transfection vehicles such
25 as liposomes can also be used to introduce the non-viral

vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The pharmaceutical composition of the present invention may be a combination of the AS-ODNs provided in the present invention. The combination is assembled and dosed as is known in the art. Further, the composition of the present invention may be a combination of one of the AS-ODNs provided in the present invention in combination with at least one other non-control AS-ODN selected from Table 1 or Table 2. This combination would have a low toxicity with a percent inhibition of approximately 25% or more.

The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles, as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the cells exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness.

5 The AS-ODN of the present invention can be administered utilizing gene therapy techniques. Generally, a DNA expression vector comprising an expressible promotor/transcriptional initiator and the AS-ODN sequence is utilized.

10 "By gene therapy" as used herein refers to the transfer of genetic material (e.g DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value.

15 Alternatively, the genetic material of interest encodes a suicide gene. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

20 Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method

25 (transfection, transduction, homologous recombination,

etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient.

10 In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* [Culver, 1998]. These genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression vehicle is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression vehicle may include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art.

20 The expression vehicle can include a promotor for controlling transcription of the heterologous material and can be either a constitutive or inducible promotor to allow selective transcription. Enhancers that may be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any non-

translated DNA sequence which works contiguously with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The expression vehicle can also include a selection gene.

5 Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in
10 Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning*
15 *Vectors and Their Uses*, Butterworths, Boston MA (1988) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central
20 nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

 The recombinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target
25 specificity and consequently, do not have to be

administered locally at the diseased site. However,
local administration can provide a quicker and more
effective treatment, administration can also be performed
by, for example, intravenous or subcutaneous injection
5 into the subject. Injection of the viral vectors into a
spinal fluid can also be used as a mode of
administration, especially in the case of neuro-
degenerative diseases. Following injection, the viral
vectors will circulate until they recognize host cells
10 with the appropriate target specificity for infection.

An alternate mode of administration can be by direct
inoculation locally at the site of the disease or
pathological condition or by inoculation into the
vascular system supplying the site with nutrients or into
15 the spinal fluid. Local administration is advantageous
because there is no dilution effect and, therefore, a
smaller dose is required to achieve expression in a
majority of the targeted cells. Additionally, local
inoculation can alleviate the targeting requirement
20 required with other forms of administration since a
vector can be used that infects all cells in the
inoculated area. If expression is desired in only a
specific subset of cells within the inoculated area, then
promoter and regulatory elements that are specific for
25 the desired subset can be used to accomplish this goal.
Such non-targeting vectors can be, for example, viral

vectors, viral genome, plasmids, phagemids and the like.

Transfection vehicles such as liposomes and colloidal polymeric particles can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Many studies show that small incremental differences in TNF- α protein levels have large effects on a variety of biological processes including viral replication (Fauci, 1996), physiological and pathological cell responses to infectious diseases (Beutler and Grau, 1993), cell death (Beutler and van Huffel, 1994; Probert et al., 1997; Talley et al., 1995), and normal cell growth and development (Arvin et al., 1996; Beutler and Grau, 1993; Tracey and Cerami, 1994). Given TNF- α 's pivotal role in disease and normal development, complete interruption of TNF- α expression is not desirable. Thus, molecular tools, such as AS-ODNs, which modulate, as opposed to eliminate gene expression, provide optimal gene regulation.

As show herein in the Example, ORF4-PE (a phosphorothioate derivative of ORF4; SEQ ID No:4) significantly reduces TNF- α mRNA levels by greater than 80% and protein levels by approximately 60% in stimulated U937 cells. A greater reduction of TNF- α mRNA compared

to protein levels is not unexpected as TNF- α has a short half-life and thus, rapid mRNA turnover (Zheng and Specter, 1996). ORF4-PE was sequence specific, efficacious in different cell types, under different
5 stimulatory conditions and did not influence the gene expression of another proinflammatory cytokine, IL-6. Further study showed that ORF4-PE, alone, does not induce TNF- α expression in U937 or PBMC (data not shown), likely due to the lack of CpG moieties and G quartets which
10 encode domains that may stimulate immune cells (Hartmann et al., 1996; Krieg et al., 1997; Krieg et al., 1996). Thus, the present invention which provides for efficient regulation of TNF- α gene expression can be achieved by using ODNs targeting exon sequences flanking donor sites.
15 Several reports show a reduction of TNF- α levels in vitro using either an antisense approach (Arima et al., 1997; Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Liang et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996; Yang et al., 1993) or a formation of
20 triplex DNA complexes (Aggarwal et al., 1996). However, in these studies, AS-ODN concentrations ranging from 2-20 μ M were required to inhibit TNF- α expression. Most studies targeting the highly conserved AUG sequence of TNF- α , assumed that this domain is the most efficient
25 site for interruption of translation. Indeed, this

approach indicates that antisense molecules directed at the 5' start region, when efficiently delivered to cells, can effectively reduce TNF- α levels (Rojanasakul et al., 1997). However, a comparison of ORF4-PE with the most efficient TNF- α -specific ODNs reported to date, which targeted the 5' AUG start region (Hartmann et al., 1996; Rojanasakul et al., 1997), showed that ORF4-PE was unexpectedly approximately 2.5-fold more efficacious at reducing TNF- α levels (Table 2). An AS-ODN complementary to the 5' AUG region of TNF- α (ORF1; SEQ ID No:1) was equally inefficient at reducing TNF- α levels (Fig. 1), suggesting that under these experimental conditions, exon sequences upstream of donor splice sites are domains that are highly vulnerable to ODN actions.

The mechanism by which ORF4-PE significantly inhibits TNF- α levels is unclear. ORF4-PE, however, is 100% complementary to the exon sequence (exon #2) flanking the donor splice site and thus, may hybridize with higher efficiency to the exon sequence, compared to the U1 small nuclear ribonucleic acid (snRNA), thereby competitively interrupting spliceosome formation and subsequent splicing (Staley and Guthrie, 1998). An examination of the upstream nucleotide sequence flanking the donor splice site of exon #2 reveals a non-consensus, variant sequence. Encoded within the exon immediately

upstream of the donor splice site (3' end of the 2nd exon) is the sequence 5'TCA3' whereas the sequence 5'A/C AG3' more frequently occurs at approximately 70, 62 and 80% respectively, at these positions (Hertel et al., 1997; 5 Padgett et al., 1986; Tarn and Steitz, 1997). This same position within the third exon of TNF- α however, is fully conserved which may explain why ORF6, which targets the exon sequence upstream of the donor site flanking exon #3, is less effective than ORF4.

10 Failure to recognize short internal exons by the spliceosome may be due to juxtaposition of adjacent 3' and 5' splice sites of internal exons, thereby creating steric hindrance and improper spliceosome/splice site interactions (Dominski and Kole, 1991). Both internal 15 exons of TNF- α (exon #2- 46 bp and exon #3- 48 bp) (accession # M16441: Genbank) are less than 50 bp and thus, may be subject to exon skipping (Dominski and Kole, 1991). Given the rarity of short internal exons in eukaryotic genes (less than 4%) (Hawkins, 1988) and their 20 susceptibility to exon skipping, the addition of competing AS-ODNs would further impede efficient mRNA splicing. In addition, exon skipping can be induced by improper recognition of weak donor splice sites (Dominski and Kole, 1991). Indeed, encoded within the second exon 25 flanking the donor splice site of human TNF- α is a

variant sequence that may be competitively inhibited by ORF4-PE. In either event, the exon skipping or direct inhibition of splicing would lead to decreased levels of TNF- α mRNA.

5 An alternative possibility is that ORF4-PE hybridizes with high affinity to processed mRNA, activating RNase H (Wagner, 1994). Mfold RNA modeling (Jacobson and Zuker, 1993) of human TNF- α mRNA (data not shown) however, shows that the domain complementary to
10 ORF4-PE is predicted to exist as a double stranded structure. Previous studies indicate that double-stranded nucleic acid structures do not provide highly stable targets for antisense AS-ODNs (Lima et al., 1992; Thierry et al., 1993). Thus, although secondary structure
15 of mRNA may limit AS-ODN-mediated actions (Laptev et al., 1994; Mishra et al., 1996; Mishra and Toulme, 1994), it is unexpected to find that ORF4-PE is binding to processed TNF- α mRNA and therefore activating RNase H.

20 Finally, although Lipofectin was found to decrease TNF- α production in U937 cells by approximately 15%, use of this carrier significantly increased the efficiency of ORF4, ORF4-PE and ORF6. These studies indicated that all AS-ODNs required a final positive net charge (as determined by the ratio of Lipofectin to ODN (Lappalainen
25 et al., 1997) in order to effectively reduce gene

expression. Specifically, Lipofectin concentrations of 10, 5 and 1 $\mu\text{g/ml}$ were mixed with ORF4 (1 μM) with 10 μg of Lipofectin having the highest degree of efficiency (data not shown). These findings are similar to previous reports of Lipofectin-enhanced cellular uptake of AS-ODNs (Hartmann et al., 1996; Lappalainen et al., 1997; Zelphati and Szoka, 1996).

These studies demonstrate that exon sequences upstream of donor splice sites within small internal exons of a naturally occurring gene, constitute domains that are highly susceptible to AS-ODN-mediated inhibition of gene expression. In addition, a highly efficient antisense AS-ODN, ORF4-PE (SEQ ID No:4), was designed which is useful *in vitro* and *in vivo* in models which study TNF- α dysregulation as well as therapeutically.

The above discussion provides a factual basis for the use of AS-ODNs that can be used in low concentrations to regulate TNF- α production in inflammatory responses. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying Figures and Tables.

EXAMPLES

METHODS:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual Series*, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.)

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition),
5 Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunassays In general, ELISAs as described herein are employed to assess the TNF- α levels. ELISA assays
10 are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively
15 described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as
20 Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989

Cell culture and primary cell preparations. The human promonocytic cell line, U937 was obtained from American Type Culture Collection, (ATCC CRL 1593.2; batch
25 F12641) (12301 Parklawn Drive, Rockville, MD 20852, USA)

and was cultured as previously outlined (Chen et al., 1997). Murine monocyte cells, IC-21 (ATCC TIB 186) were maintained at an approximate density of 2.0×10^5 cells/ml in 10 mM HEPES buffered, RPMI media supplemented with 10% FBS. Human peripheral blood mononuclear cells (PBMC) and macrophages were purified from whole blood obtained from healthy volunteers as previously outlined (Power et al., 1995). Briefly, to obtain primary human macrophages, PBMC were cultured for 3 d. in RPMI 1640 media supplemented with 20% heat-inactivated FBS and 50 units of penicillin and 50 μ g of streptomycin/L. Non-adhering cells were removed by washing with RPMI media and adhering monocytic cells were removed and cultured at a density of 2.0×10^5 cells/ml for seven days in RPMI supplemented with 20% FBS and antibiotics. All cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO₂.

Design and synthesis of AS-ODNs. AS-ODNs were generated using the computer DNA modeling program PrimerSelect (DNASTAR) which designs AS-ODNs based on optimal free energy (ΔG), low dimer formation, low hairpin formation and high thermal stability. AS-ODNs were designed to target exon sequences upstream of the donor (5') and downstream of the acceptor (3') splice sites and other regions throughout the open reading frame.

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(ORF) of human TNF- α (accession # M10988: GenBank) and were assessed for sequence similarity with other non-TNF- α mRNAs (GenBank). TNF- α mRNA numbering was defined such that A in the AUG start codon was position +1. AS-ODNs
5 were chosen if they met the following requirements: size between 16 and 30 mer; low hairpin formation (≤ 3 base pairs); low dimer formation (≤ 3 consecutive bases) and melting temperatures above 45 °C. All AS-ODNs were synthesized using standard phosphoramidite methods at
10 0.05 or 0.2 μ mol scales and were HPLC purified by the manufacturer (Life Technologies, Mississauga, ONT).

Cell culture stimulation and AS-ODN treatment.

Immortalized monocytic cells (U937 (human) or IC-21 (murine)) were cultured to approximately 80% confluency
15 and were seeded at a density of 400,000 cells/ml (U937) or 200,000 cells/ml (IC-21) in a 96 well plate in OPTI-MEM serum reduced media (pH 7.4) supplemented with 5% FBS without antibiotics. Human PBMC were seeded at a density of 500,000 cells/ml. Serum levels were reduced to 5% as
20 recommended for Lipofectin use by the manufacturer. AS-ODNs (1 μ M, 100 and 10 nM) were mixed with Lipofectin (10 μ g/ml) (Life Technologies, Mississauga, Ontario, Canada) and added to the cell cultures for three hours. The cells were subsequently stimulated for 1 h with 10
25 ng/ml of phorbol-12-myristate-13-acetate (PMA) and 5

5 μ g/ml phytohemagglutinin (PHA). The cells were then washed once with OPTI-MEM media supplemented with 5% FBS. AS-ODN/Lipofectin mixtures equal to the initial dosage were then added to each respective well. Since stimulated macrophages display maximum TNF- α mRNA levels at 3 hours, and protein formation at 3 to 4 hours (Zheng and Specter, 1996), all cells treated with AS-ODNs were incubated for 4 hours at 37 °C and supernatants were collected, centrifuged at 700 x g for 5 minutes and analyzed for TNF- α by ELISA. To determine AS-ODN cytotoxicity, U937 cells were treated with 5, 1, and 0.1 μ M of AS-ODNs. AS-ODN-mediated cell death was determined both in the presence and absence of Lipofectin by measuring cell proliferation and viability (as measured by trypan blue exclusion) at 4, 8, and 24 hours. All antisense screening experiments were performed in triplicate, a minimum of three times.

LPS treatment of primary macrophages. Primary macrophage cultures (200,000 cells/ml) were maintained in complete RPMI media supplemented with 20% FBS and antibiotics for 7 days following purification. Cell cultures were washed once with RPMI media and then suspended in OPTI-MEM serum-reduced media supplemented with 5% FBS the day prior to the experiment. Macrophage cultures were treated with Lipofectin-delivered AS-ODNs

for 3 hours, washed once with OPTI-MEM media supplemented with 5% FBS and stimulated for 1 hour with 1 µg/ml lipopolysaccharide (LPS) (*E. coli* type 055:B5) (Sigma) and AS-ODNs were administered as outlined above.

- 5 Following 4 hours incubation, the supernatants were collected and analyzed by ELISA.

Quantitative immunoassay for cytokines. TNF-α levels in tissue culture supernatants were determined by a sandwich ELISA as previously reported (Chen et al.,
10 1997). Human IL-6 and murine TNF-α levels were quantified using sandwich ELISA according to the manufacturer (Pharmingen). Serial doubling dilutions of human or murine recombinant TNF-α (1250 to 4.5 pg/ml) or IL-6 (2500 to 19.5 pg/ml) was used to generate standard
15 curves. For all experiments, values are corrected for the presence of Lipofectin and are presented as mean ± SD (n=3).

Total RNA extraction and RT-PCR. Total cellular RNA was prepared from approximately 1 X 10⁶ cells as
20 previously described (Gough, 1988). Pilot studies were performed comparing different PCR cycle number and input RNA concentrations to ensure linear amplification of template occurred. RT-PCR amplification was within linear range when 2 µg of total RNA was reverse
25 transcribed into cDNA (Pharmacia, Mississauga, ONT).

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followed by PCR amplification of 2 μ l of cDNA product using either TNF- α or GAPDH cDNA specific primers (Chen et al., 1997; Wesselingh et al., 1993) for 25 cycles at 95°C denaturation (60 s), 60°C annealing (60 s) and 72 °C extension (60 s). These conditions confirmed previous findings by applicants (Chen et al., 1997; Wesselingh et al., 1993). Products were separated by agarose gel electrophoresis (1.4%), transferred to a nylon membrane and probed using a randomly labeled 32 P-dCTP human TNF- α cDNA (Wang et al., 1985) or human GAPDH (Chen et al., 1997). Densiometric analysis of RT-PCR products was performed using the public domain program NIH Image (Ver 1.60).

Statistical analysis. Results were statistically analyzed by two-tailed Student's t-test.

RESULTS

Exon sequences immediately upstream of donor splice sites of TNF- α are highly susceptible to AS-ODN-mediated inhibition. Recent evidence suggests that TNF- α production can be regulated by antisense AS-ODNs, however, AS-ODN concentrations ranging from 2-20 μ M are required to reduce TNF- α levels in cells (Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996). At a concentration of 1 μ M or less, applicants examined the

efficiency of AS-ODNs targeting exon sequences upstream of donor sites and downstream of acceptor splice sites as well as other regions within the TNF- α mRNA (Table 1). These studies unexpectedly show that AS-ODNs targeting

5 exon sequences upstream of the donor splice site of exon 2 (ORF4; SEQ ID No:4) and 3 (ORF6; SEQ ID No:6) reduced TNF- α levels in PMA/PHA stimulated U937 cells (Fig. 1, Table 1). TNF- α levels in stimulated U937 cells were reduced by $62 \pm 7\%$ ($p < 0.001$) by ORF4 (SEQ ID No:4) and $51 \pm 9\%$ ($p < 0.005$) by ORF6 (SEQ ID No:6) (Fig. 1, Table 1).

10 In contrast, AS-ODNs targeting exon sequences downstream of the acceptor sites of exon 2 (ORF3; SEQ ID No:3) and 3 (ORF5; SEQ ID No:5) or AS-ODNs targeting other TNF- α mRNA domains, including the 3' UTR UA rich region did not

15 significantly reduce TNF- α levels (Fig. 1, Tables 1, 2). AS-ODNs designed to complement the 5' AUG start site of human TNF- α (Rojanasakul et al., 1997) were not as efficacious as ORF4 (SEQ ID No:4) or ORF6 (SEQ ID No:6) under the same conditions (Fig. 1, Table 1) and

20 mismatched versions of ORF4 ($n=2$) did not significantly inhibit TNF- α production (Table 1). In addition, a 21 mer AS-ODN, O-8433 (SEQ ID No:23) that targets the HIV-1 tat gene, was used to assess for non-specific AS-ODN effects. O-8433 did not significantly affect supernatant

25 TNF- α levels in stimulated U937 cells (Table 1).

Since ORF4 and ORF6 displayed the greatest inhibition of TNF- α synthesis, these antisense oligonucleotide molecules were further analyzed. In addition to 1 μ M of ORF4 and ORF6 significantly reducing TNF- α levels, 100 and 10 nM of ORF4 reduced levels by 40 \pm 9% and 19 \pm 7% respectively and 100 and 10 nM of ORF6 reduced TNF- α levels by 26 \pm 8% and 18 \pm 9% respectively (Fig. 2a).

To ensure that 1 μ M concentrations of AS-ODN were not toxic to U937 cells, AS-ODN concentrations as high as 5 μ M were added to U937 cells which were subsequently tested for proliferation and viability. Five μ M ORF4 had no effect on cell proliferation or viability at 4, 8 or 24 hours (Fig. 2b). Cells treated with AS-ODNs delivered by Lipofectin showed similar results up to 24 hours. After 24 hours treatment however, Lipofectin-treated cells showed significant cell death, presumable due to Lipofectin cytotoxicity (Bell et al., 1998; Yagi et al., 1993) (data not shown).

20

Detailed analysis of AS-ODNs targeting exon sequences flanking donor and acceptor splice sites. Since AS-ODNs complementary to the exon sequences upstream of the donor splice site of exons 2 and 3 of TNF- α significantly inhibited TNF- α production, adjacent nucleic acid domains

of the exons were examined in greater detail (Table 2). Specifically, AS-ODNs (n=10) were designed to target regions spanning the small internal exons (exons 2 and 3) of human TNF- α . All AS-ODNs were partially

5 phosphorothioated (approximately 30%) and ORF4 (SEQ ID No:4) was partially phosphorothioated (ORF4-PR) or phosphorothioated at 3 bases on each end of ORF4 (SEQ ID No:4, ORF4-PE) in order to increase nuclease stability (Table 1) (Uhlmann et al., 1997). Only AS-ODNs targeting

10 exon sequences upstream of the donor splice site, independent of their size, significantly reduced TNF- α production (Table 2). Conversely, AS-ODNs targeting the downstream exon sequences of the acceptor site did not reduce TNF- α levels to the same extent. Of all AS-ODNs

15 tested in U937 cells, ORF4-PE was the most efficacious (65 \pm 5%) (Table 2). In contrast, ORF4-PR, which was randomly phosphorothioated throughout its 29 mer sequence, reduced TNF- α levels by 42 \pm 5% which was significantly less (p<0.001) than ORF4-PE (Table 2)

20 suggesting that site(s) of phosphorothioation may be critical determinants of AS-ODN efficiency.

ORF4-PE (SEQ ID No:4) dose-dependently reduces TNF- α mRNA levels in stimulated U937 cells. To determine the extent

25 to which ORF4-PE influenced TNF- α mRNA levels RT-PCR was

performed using primers that amplified a segment of TNF- α spanning exons 2, 3 and 4. RT-PCR products, confirmed by Southern analysis, showed that ORF4-PE dose-dependently reduced the levels of the correctly processed TNF- α mRNA.

5 Densiometric analysis of RT-PCR products confirmed that as the concentration of ORF4-PE increased, TNF- α mRNA detection decreased (Fig. 3). Interestingly, an additional RT-PCR product, smaller than expected (430 bp), was present in samples treated with 1 μ M ORF4-PE
10 (data not shown). Although the source of this PCR product is unknown, this band may be the result of a cryptic splicing event (Hodges and Crooke, 1995). However, larger intermediary RNA species were not observed, perhaps due to rapid degradation of improperly
15 spliced pre-mRNA (Khoury et al., 1979).

ORF4-PE (SEQ ID No:4) efficiency in U937, human PBMC and primary macrophages and immortalized murine monocytes.

Since phosphorothioation of the end sequences of ORF4-PE
20 did not impede its efficiency in stimulated U937 cells, this antisense molecule was used in all subsequent experiments. Phosphorothioation of ORF4 could potentially introduce non-specific effects (Hartmann et al., 1996), therefore, the specificity of ORF4-PE was
25 further analyzed. ORF4-PE (5 μ M), in the absence of

Lipofectin, was not toxic to U937 cells (data not shown) and displayed a dose response similar to that of ORF4 where 1 μ M, 100 and 10 nM reduced TNF- α levels in stimulated U937 cells by $65 \pm 5\%$, $36 \pm 7\%$ and $23 \pm 8\%$ respectively (Fig. 4a). To determine whether ORF4-PE influenced other inflammatory cytokine levels, IL-6 levels were measured in supernatants from stimulated U937 cells treated with ORF4-PE. ORF4-PE doses of 1 μ M, 100 and 10 nM did not significantly affect IL-6 levels in the supernatants of stimulated U937 cells (Fig. 4a).

To determine the efficiency of ORF4-PE in primary human cells under different stimulatory conditions, primary cells were treated with ORF4-PE and stimulated with PMA/PHA (10 ng/5 μ g/ml; PBMC) or with LPS (1 μ g/ml; macrophages). ORF4-PE (1 μ M) treatment of PBMC cultures significantly decreased PMA/PHA TNF- α gene expression by $62 \pm 9\%$ ($p < 0.001$) (Fig 4b). ORF4-PE (1 μ M) treatment of LPS-stimulated primary macrophages significantly decreased TNF- α levels by $73 \pm 8\%$ ($p < 0.00004$) (Fig. 4b). In both PBMC and primary macrophages, 100 and 10 nM ORF4-PE showed a trend of dose-dependent reduction of TNF- α levels (Fig. 4b). All PBMC ($n=5$) and primary macrophage cultures ($n=5$), were susceptible to ORF4-PE treatment with inhibition of TNF- α ranging approximately from 50-70% in PBMC cultures and from 70-85% in primary

macrophages (data not shown). ORF4-PE efficiency of TNF- α reduction was approximately 10-15% greater in primary macrophage cultures compared to PBMC or U937 cells, possibly due to active AS-ODN/Lipofectin phagocytosis by
5 primary macrophages (Chaudhuri, 1997; Iversen et al., 1992). Finally, since the TNF- α gene sequence is highly conserved across species, the relative cross-species efficiency of ORF4-PE was tested in murine monocytes treated with ORF4-PE and stimulated with LPS. ORF4-PE (1
10 μ M) significantly reduced murine TNF- α levels in LPS-stimulated cells by $48 \pm 8 \%$ ($p < 0.009$) (Fig. 4b). Thus, in both human and murine cells, exon sequences upstream of the donor splice site of the 2nd exon of TNF- α are highly susceptible to AS-ODN actions.

15 In summary, exon sequences upstream of splice sites play a critical role in mRNA processing. Correct mRNA processing is dependent on spliceosome interactions with these sites. Using antisense oligodeoxynucleotides (AS-ODNs), these and other sequences of the proinflammatory
20 tumor necrosis factor alpha (TNF- α) gene were targeted because it is multiply spliced and has been difficult to regulate with AS-ODNs in the past. AS-ODNs targeting exon sequences upstream of the donor splice sites of internal exons 2 (ORF4) and 3 (ORF6) significantly
25 reduced TNF- α levels in stimulated U937 cells by $62 \pm 7\%$

and $51 \pm 9\%$, respectively, in a dose-dependent manner but did not affect IL-6 levels (see Tables). In contrast, AS-ODNs targeting the exon sequences downstream of the acceptor splice sites of exon 1, 2 and 3 failed to reduce TNF- α levels significantly under the same conditions. End-phosphorothioated ORF4 (ORF4-PE) significantly reduced TNF- α mRNA levels by greater than 80% ($p < 0.001$) and protein levels by 60% ($p < 0.001$) in U937 cells. ORF4-PE reduced newly synthesized TNF- α protein levels by greater than 80% in LPS-stimulated human macrophages, by greater than 60% in PMA/PHA-stimulated human PBMC and by approximately 50% in LPS-stimulated murine monocytes. These results show that exon sequences flanking donor splice sites provide highly vulnerable target domains for antisense inhibition of TNF- α gene expression.

Throughout this application, various publications, including United States patents, are referenced either by number or by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology

which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

Table 1. ODN sequence, target domain and % inhibition of TNF- α .

AS-ODN	Sequence ¹ / SEQ ID No:	Mer	TNF- α Position ²	% inhibition ³ (mean \pm SD)
ORF1	GCC AGC TCC ACG TCC CGG ATC ATG CTT TCA SEQ ID No:1	30	+9- +38	25 \pm 5
ORF2	GGC TGA GGA ACA AGC ACC GCC TGG AG SEQ ID No:2	26	+78- +103	16 \pm 6
ORF3	GAC TCT TCC CTC TGG GGG CCG ATC ACT CCA SEQ ID No:3	30	+159- +188	25 \pm 5
ORF4	CTG ACT GCC TGG GCC AGA GGG CTG ATT AG SEQ ID No:4	29	+205- +233	62 \pm 7**
ORF5	TCG GGG TTC GAG AAG ATG ATC TGA CTG C SEQ ID No:5	28	+226- +253	14 \pm 4
ORF6	CCA CAT GGG CTA CAG GCT TGT CAC TCG SEQ ID No:6	27	+251- +277	51 \pm 9*
ORF7	GCT TGA GGG TTT GCT ACA ACA TGG GCT ACA SEQ ID No:7	30	+264- +293	18 \pm 6
ORF8	GGC CCG GCG GTT CAG CCA CTG GAG SEQ ID No:8	24	+304- +327	36 \pm 4
ORF9	CAC GCC ATT GGC CAG GAG GGC ATT GG SEQ ID No:9	26	+326- +351	30 \pm 3
ORF10	AGG TAC AGG CCC TCT GAT GGC ACC ACC AG SEQ ID No:10	29	+370- +398	25 \pm 5
ORF11	CCT GTC TTC TTG GGG AGC GCC TCC TC SEQ ID No:11	26	+40- +65	22 \pm 6
ORF14	CTG GGG CCC CCC TGT CTT CTT GGG GA SEQ ID No:12	26	+50- +75	15 \pm 8
ORF15	GCC TGG AGC CCT GGG GCC CCC CTG TC SEQ ID No:13	26	+60- +85	19 \pm 7
ORF16	ACA AAG CAC CGC CTG GAG CCC TGG GG SEQ ID No:14	26	+70- +95	24 \pm 6
ORF17	GGA AGG AGA AGA GGC TGA GGA ACA SEQ ID No:15	24	+92- +115	22 \pm 5
ORF18	TGC CAC GAT CAG GAA GGA GAA SEQ ID No:16	21	+106- +126	25 \pm 8
ORF21	GCA GCA GGA AGA AGA GCG TGG TG SEQ ID No:17	23	+132- +154	28 \pm 4
O-3'UTR	AAT AAT AAA TAA ATA ATA AAT AAT CAC AAG SEQ ID No:18	31	+1310- +1339	21 \pm 5

ODN 5' A⁵

ODN 5' A ⁵	CAT GCT TTC AGT CAT	15	5' AUG start	26 ± 8
	SEQ ID No:19			
ODN 5' B ⁶	TGT GCT CAT GGT GTC TTT	18	5' AUG start	27 ± 9
	SEQ ID No:20			
Controls				
ORF4MM1 ⁴	CTG ACA TCC TGG GCC CCA GGG CTG ATT AG	29	+205- +233	22 ± 9
	SEQ ID No:21			
ORF4MM2 ⁴	CTG ACT GCC TGC TCC AGA GGG CTG ATT	27	+207- +233	28 ± 8
	SEQ ID No:22			
O-8433	ATC GTC CGG ATC TGT CTC TGT	21	HIV-1 Tat	19 ± 4
	SEQ ID No:23			

1. Antisense ODN sequences are shown in a 5' to 3' direction.
 2. Positions are numbered relative to the 5' AUG sequence of TNF- α .
 3. U937 cells were treated with 1 μ M ODN and percent inhibition of TNF- α was corrected for the presence of Lipofectin.
 4. Mismatches of bases within ORF4 are denoted in bold.
 5. Hartmann et al. 1996.
 6. Rojanasakul et al. 1997
- ** ($p \leq 0.001$).

Table 2. ODNs sequence, target site within the second or third exon and % inhibition of TNF- α .

AS-ODN	Sequence ¹ / SEQ ID No:	Mer	TNF- α position ²	Flanking ³ splice site	% inhibition ⁴ (mean \pm SD)
exon 2					
ORF4-PE	CTG ACT GCC TGG GCC AGA GGG CTG ATT AG	29	+205- +233	donor	65 \pm 5**
	SEQ ID No:4				
ORF4-PR	CTG ACT GCC TGG GCC AGA GGG CTG ATT AG	29	+205- +233	donor	42 \pm 5*
	SEQ ID No:4				
O4.5	GAT TAG AGA GAG GTC CCT GGG	21	+190- +210	acceptor	32 \pm 6
	SEQ ID No:24				
O4.10	TGG GCC AGA GGG CTG A	16	+209- +224	mid exon	31 \pm 7
	SEQ ID No:25				
O4.4	AGG GCT GAT TAG AGA GAG GTC	21	+195- +216	mid exon	31 \pm 8
	SEQ ID No:26				
O4.1	TGC CTG GGC CAG AGG GCT GAT TAG	24	+205- +228	donor	43 \pm 5*
	SEQ ID No:27				
O4.2	CTG ACT GCC TGG GCC AGA GGG CTG	24	+210- +233	donor	42 \pm 4*
	SEQ ID No:28				
O4.3	ACT GCC TGG GCC AGA GGG CTG	21	+210- +230	donor	39 \pm 5*
	SEQ ID No:29				
O4.7	TTC GAG AAG ATG ATC TGA CTG	21	+227- +247	donor	44 \pm 8*
	SEQ ID No:30				
exon 3					
O4.6	GAA GAT GAT CTG ACT GCC TGG	21	+222- +242	acceptor	28 \pm 4
	SEQ ID No:31				
O4.8	GGG GTT CGA GAA GAT GAT	18	+233- +251	acceptor	34 \pm 5
	SEQ ID No:32				
O4.9	CTT GTC ACT CGG GGT TCG	18	+244- +261	mid exon	32 \pm 4
	SEQ ID No:33				

1. Antisense ODN sequences are shown in a 5' to 3' direction. Phosphorothioated nucleotides are denoted in bold font.
2. Positions are numbered relative to the 5' AUG sequence of TNF- α .
3. ODNs were designed to target exon sequences flanking donor or acceptor sites of the internal exons of TNF- α .
4. U937 cells were treated with 1 μ M ODN and % inhibition of TNF- α was corrected for the presence of Lipofectin.

* (p \leq 0.01) ** (p \leq 0.001).

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